Hydrogen Ion Equilibria of the Genetic Variants of Bovine β-Lactoglobulin

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The hydrogen ion equilibria of β-lactoglobulins A, B, and C have been examined. The resulting titration curves can be accounted for in terms of the normal ionization of all groups, with the exception of two histidines in the C variant and two carboxyls in all three of the variants. Conformational changes at pH 4.5–6 and 6.5–9 release these abnormal groups to ionization.

The dissociation of hydrogen ions from β-lactoglobulin was first studied in detail by Cannan *et al.* (1) who reported the effects of temperature, concentration of KCl, concentration of protein, and the addition of formaldehyde on the dissociation curve of the protein. Nozaki *et al.* (2) and Tanford and Nozaki (3) later showed that the isoionic point of β-lactoglobulin decreases when KCl and $CaCl_2$ are added, and interpreted these results in terms of K^+ and Ca^{++} binding by the isoionic protein. In addition, they concluded (4), on the basis of optical rotation and the titration curve of β-lactoglobulin, that a reversible change in the protein configuration occurs around pH 7.5 at 25°. This change is accompanied by the reversible release to ionization of two carboxyl groups from the interior of the molecule.

Following the report of Aschaffenburg and Drewry (5, 6) on the existence of two genetically different bovine β-lactoglobulins, A and B, Tanford and Nozaki (3) compared the isoionic points, titration curves, optical rotatory dispersion, and ultraviolet absorption of these two variants. They found that the only difference in the two proteins was in the number of titratable carboxyl groups, β-A having two more of these groups than β-B per 35,500 dalton isoelectric dimer. This difference was later characterized by Townend (7) as being due to the substitution of two aspartic acid residues in the primary structure of β-A for two glycines in β-B. The proteins exhibited similar behavior in their optical rotation, in the displacement of their isoionic points by addition of KCl, and the occurrence of a configurational change at pH 7.5 as reflected in a steepening of the titration curve at that pH.

More recently a third genetic variant of β-lactoglobulin, β-C, was discovered by Bell (8). This was found to differ from β-B by the substitution in the C variant of a histidine residue for that of a glutamic acid (or glutamine) in the amino acid sequence of β-B (9, 10). The present experiments were undertaken to establish whether this substitution involved the free carboxylic acid or the neutral carboxamide. In this paper we report these results, and compare the titration curves of the three genetic variants of this protein and describe a new conformational transition of the β-lactoglobulins which was discovered in the course of the work.

EXPERIMENTAL PROCEDURE

β-Lactoglobulins A, B, and C were prepared by the method of Aschaffenburg and Drewry (11) from milks of cows homozygous for each of the three variants (12). The preparation of β-C was

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modified (9, 10) because of its greater solubility than the other two proteins. The purity of each protein was verified by DEAE-column chromatography (9, 12).

Stock solutions were prepared by dissolving the protein in 0.15 M KCl and clarifying the solutions in a Dannley pressure filter funnel. Protein concentrations were determined spectrophotometrically at 280 mμ, using an absorptivity value of 0.94 liter per gram (2, 3). The protein concentrations so determined were verified by dry weight determinations after making the appropriate corrections for salt content. A second set of stock solutions was prepared by passing a solution of each protein through a mixed-bed ion exchange resin column according to the procedure described by Dintzis (13). Removal of ions other than hydrogen and hydroxyl was checked by conductivity measurements. The isoionic protein solutions were used for the titrations except in the region below pH 2.5, where more concentrated solutions are required (3). They were also used to determine the effect of added KCl on the isoionic points of the β-lactoglobulins. Solutions made directly in 0.15 M KCl were employed to determine the hydrogen ion dissociation curves for the denatured proteins.

Aliquots of the stock solutions were diluted to a constant volume and constant ionic strength of 0.15 M with standard HCl, KOH, and KCl. The pH measurements were made on these at 25° in an atmosphere of purified nitrogen using a Radiometer pH meter model 4.[2] The pH meter was standardized with a buffer of appropriate pH before each experimental determination. All additions to the protein solutions were made with Hamilton gas-tight syringes equipped with Chaney adaptors.

Alkaline denatured β-lactoglobulins were prepared by adding sufficient 5 N KOH to the native proteins to bring the pH of the stock solutions to 11.1. These solutions were allowed to stand for 10 minutes at 25° and then diluted with KCl and HCl, after which the pH was measured.

For experiments on the urea denatured samples, aliquots of protein solutions, made up in 0.15 N KCl, were lyophilized and their weights were determined and corrected for salt content. A solution of 8 M urea without KCl was then added to the dried aliquots up to their original volume and the resulting solutions were allowed to stand for 30 minutes at 25°. Each aliquot of the urea-denatured protein solution was diluted to a constant volume with standard HCl and KCl, and the pH was measured. Appropriate corrections were made for the specific volumes of protein and salt. In this case, the pH region of interest was the acid region only.

The molecular weight for each of the β-lactoglobulin variants was taken as 35,500 (14), which is the kinetic unit of these proteins in the isoelectric region. All results reported in this paper are related to that molecular weight, even though β-lactoglobulin is known to undergo polymerization (15–17) and dissociation (18–20) reactions under certain conditions.

[2]It is not implied that the U.S. Department of Agriculture recommends the above company or its products to the exclusion of others in the same business.

RESULTS

Isoionic points. When solutions of each of the three genetic variants were passed through a Dintzis deionization column (13), crystals of β-A separated almost immediately, but β-B crystallized slowly and β-C remained dissolved. The pH's of 2.0 gm per liter isoionic solutions were 5.35 for β-A, 5.41 for β-B, and 5.39 for β-C; these values can be considered as applicable to higher concentrations also, since it has been shown by Treece *et al.* (21) that the expected pH rise due to progressive ionization of the protein occurs only at concentrations below 1 gm per liter in the case of the β-lactoglobulins. Addition of KCl lowered the pH of all three genetic variants, as shown in Fig. 1a. At an ionic strength of 0.15, the difference in isoionic points is found to be 0.095 pH unit between β-A and β-B, in good agreement with Nozaki *et al.* (2). This would correspond to a difference of 1.4 titratable groups per 35,500 dalton molecule. While the pH values of β-C at various salt concentrations are similar to those of β-B, the decrease upon addition of salt appears to be somewhat less in this genetic variant. β-C has a slightly lower pH than β-B in the deionized state, and this relationship becomes reversed above 0.1 M KCl. If, as concluded by Nozaki *et al.* (2), these displacements in pH with addition of salt are the result of potassium binding, the number of ions bound per protein molecule, ν_{K^+}, can be calculated using the equation of Scatchard and Black (22):

$$\nu_{K^+} = \frac{\Delta pH}{0.868w}, \qquad (1)$$

where ΔpH is the difference in pH between the deionized solution and that at the given

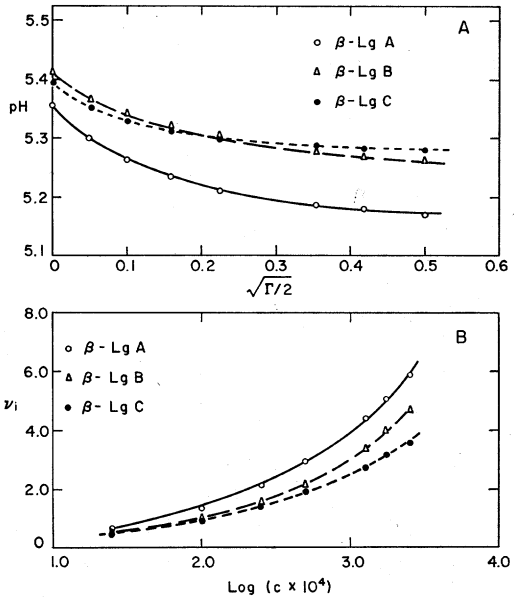

FIG. 1. Interaction of the β-lactoglobulins with KCl. (A) Change in pH with increasing ionic strength (Γ/2). (B) Apparent number of K^+ ions bound (ν_i) per β-lactoglobulin molecule.

salt concentration, and w is the electrostatic interaction parameter,

$$w = \frac{e^2}{2DkTb}\left(1 - \frac{\kappa b}{1 + \kappa a}\right). \qquad (2)$$

Here e is the electronic charge, D is the dielectric constant of the medium, k is Boltzmann's constant, T is the thermodynamic temperature, κ is the Debye-Hückel parameter, a the radius of closest approach of small ions to the macroion, and b is the radius of a sphere with a volume equal to the hydrated volume of the protein molecule. Using the radius of gyration of β-lactoglobulin, Rg, determined by small angle X-ray scattering, Rg = 21.6 Å (17), b is found to be 27.7 A, since $b = (3/2)^{1/3} Rg$. The values of apparent potassium ion binding given by the three isoionic genetic variants as a function of KCl concentration are shown in Fig. 1b. These are found to be in general good agreement with those published by Nozaki *et al.* (2) for a mixture of variants A and B, once the latter have been adjusted to the value of b derived from the radius of gyration. β-A is found to bind K^+ somewhat more strongly than B, and B more so than C. This is not surprising in view of the known presence in β-A of two more anionic groups than in β-B (2, 3, 7). Nozaki *et al.* (2) have postulated that the positive ions are bound at a site formed of a constellation of carboxyls, and Townend (7) has given evidence suggesting that the difference aspartic residue may indeed be present in such a cluster.

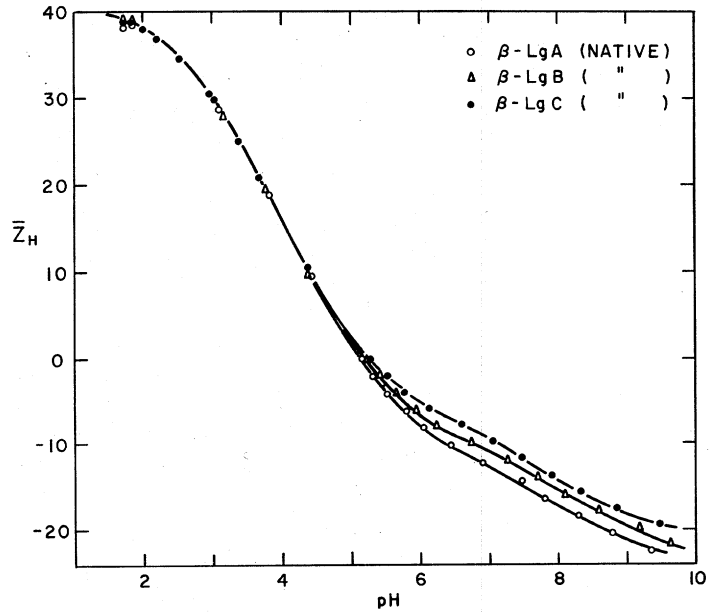

FIG. 2. Titration curves of the β-lactoglobulins in 0.15 ionic strength KCl.

TABLE I

GROUPS TITRATABLE BELOW pH 9 IN THE β-LACTOGLOBULINS (35,500 M.W.)

	β-A	β-B	β-C	pK
α-COOH	2 (2)	2 (2)	2 (2)	3.75
β,γ-COOH (Normal)	50 (52)[a]	48 (50)	48 (50)	4.66
β,γ-COOH (Anomalous)	2	2	2	7.25
Imidazole (Normal)	4 (4)	4 (4)	4 (6)	7.25
Imidazole (Anomalous)	0	0	2	
α-NH_2	2 (2)	2 (2)	2 (2)	7.80
Total cationic	(40)	(40)	(42)	

[a] The number in parentheses is the number of residues found in amino acid analysis.

Stoichiometry. Figure 2 shows the titration curves between pH 1.8 and 9.4 of the three genetic variants. Below pH 4.3, the curves are identical, all tending toward a maximal acid binding capacity of 40 H^+ per molecule. Above pH 4.5 the three curves separate, the difference in groups titrated at pH 9.4 being 2.0 between β-A and β-B and 1.6 between β-B and β-C. These differences are consistent with the differences in amino acid composition of the genetic variants (7, 9, 10). Furthermore, the titration curves of β-A and β-B are essentially identical with those published by Nozaki *et al.* (2), and the numbers of different types of titratable groups given in Table I agree with the amino acid analyses (23, 24) of these two proteins.[3]

When all groups are available to solvent, the total acid binding capacity of a protein is equal to the total number of basic groups. The value of 40 such groups in β-A and β-B is in perfect agreement with the amino acid analysis of these variants. In the case of β-C, a discrepancy occurs: amino acid analysis gives a total of 42 basic groups [due to the presence of the two substitution histidines (9, 10)], while the titration data would indicate the presence of only 40 such groups, as in the other the two variants. All three variants display the steepening of the titration curve between pH 7 and 8.5, previously reported for β-A and β-B (2); this has been shown (2) to be due to two anomalously titrating carboxyls with an apparent pK of 7.5, which are released to contact with solvent and subsequent ionization in this pH region by a conformational transition (2, 25). From the data of Fig. 2, it seems quite evident that β-C undergoes this same transition. The numbers of the various ionizable groups in β-C and their pK's were determined by analyzing the titration curve in terms of the Linderstrøm-Lang equation (26, 27):

$$\text{pH}-\log\frac{\alpha}{1-\alpha} = \text{p}K'-0.868w'\bar{Z}_H, \quad (3)$$

where α is the degree of dissociation of a given kind of group, $\bar{Z}_H$ is the net average charge of the molecule, and w' and pK' are the apparent values of the electrostatic interaction parameter and the pK of the group and differ from the intrinsic values of these constants because of the binding of ions other than protons (2). Figure 3 shows this plot for the carboxyl ionization of β-A, β-B, and β-C. The points obtained fall on a single straight line up to $\bar{Z}_H = 25$; application of Eq. 3 results in p$K' = 4.66$ and $w' = 0.040$ for all three variants in 0.15 M KCl. This is in excellent agreement with the values of 4.69 and 0.039 obtained by Nozaki *et al.* (2) for a mixture of β-A and β-B.

The identity of the three titration curves below pH 4.5 and the discrepancy by two groups between the total acid binding capacity of β-C and its amino acid composition indicate that in this genetic variant two ionizable groups remain unprotonated down to

[3] No data were obtained above pH 9.5 because of the irreversible denaturation at alkaline pH's; thus no direct information has been obtained on the titration of phenolic, sulfhydryl, ε-amino, and guanidyl groups.

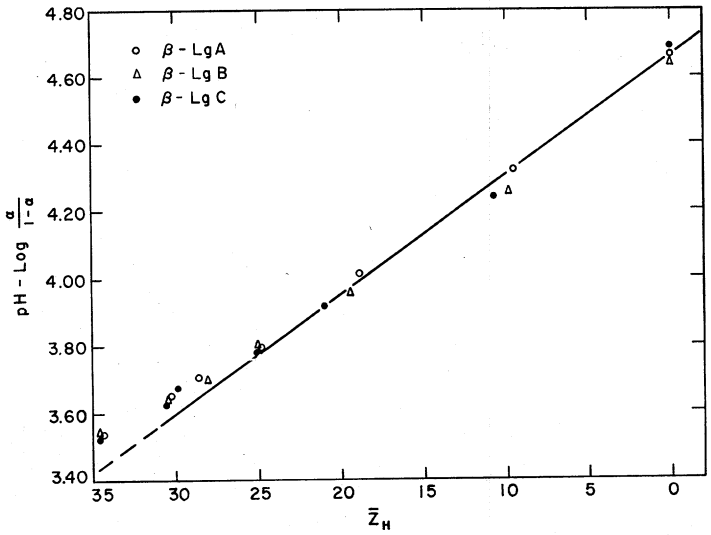

FIG. 3. Linderstrøm-Lang plot of side-chain carboxyl ionization.

the acid end of the titration curve. In order to determine the nature of these groups, titration curves were calculated by using the known amino acid composition o the C variant and the pK' values of the titratable groups as reported by Nozaki *et al.* (2) for the β-A, β-B mixture (these are listed in Table I). This approach is quite reasonable, since there are no gross differences between the hydrodynamic and optical rotatory dispersion behavior of the three genetic variants over the pH range of interest.

Since the differences in amino acid composition between β-B and β-C are the substitution of two histidines in C for two glutamic acid, or glutamine, residues in B (9, 10), the titration curve of β-C would be expected to be identical with B in all respects, except for the addition of two basic groups with pK' = 7.25 and possible loss of two acidic groups with pK' = 4.66. Titration curves were calculated, therefore, for a number of possible combinations of titratable imidazoles and carboxyls; all other groups were kept identical to their numbers in β-B with the appropriate pK's, listed in Table I. The first two curves, corresponding to the two possible amino acid analyses were: *Case 1*, 46 β and γ carboxyls and 6 imidazoles; *Case 2*, 48 β and γ carboxyls and 6 imidazoles. These are shown in Fig. 4, where the experimental points are given by the open circles. The curve for Case 1 (shown by solid line 1) runs above the experimental points, displaced from it by two to four titratable groups. Case 2 yields a curve which shifts from solid line 1 at the acid end to solid line 2 above pH 6, following the dotted line between pH 2.5 and 6. It is evident that while neither curve fits the experimental data, Case 2 agrees very well with the alkaline end of the data (above pH 6). This shows that β-C has 48 β and γ carboxyls and establishes that the amino acid substitution results in β-C having two less glutamines than β-A or β-B, their glutamic acid contents being identical.

Since the total acid binding capacity points to the protonation of two less basic groups than could be expected from the amino acid analysis, an attempt was made to fit the data with the assumption that the two substitution histidines are buried inside the hydrophobic interior of the molecule, never becoming exposed to solvent and thus unable to accept protons. The resulting calculated curve (Case 3) follows solid line 2 of Fig. 4 up to pH 4.5 in good agreement with the experimental points; above pH 4.5, however, the calculated curve (dashed line) falls below the experimental points and reaches a difference of two bound protons at pH 6.5 and rejoins solid line 2 and the experimental points above pH 9. Comparison of these

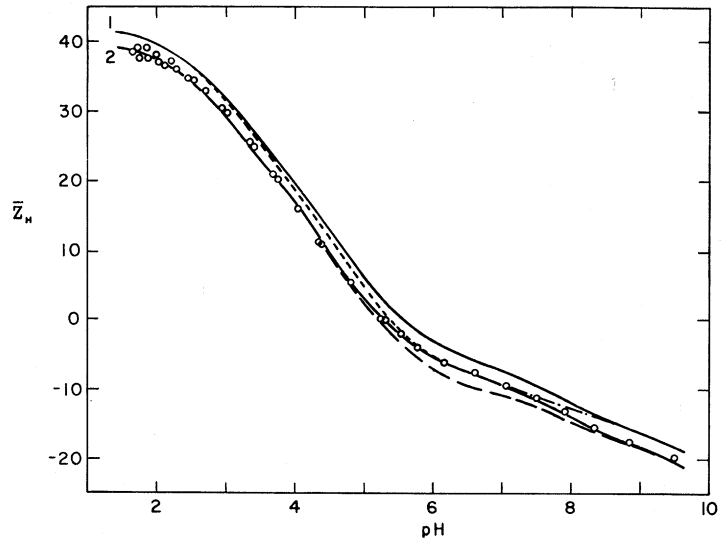

FIG. 4. Calculated titration curves of β-C. Circles are experimental points; the significance of the various types of curves is explained in the text.

three calculated curves with experimental data shows that, while above pH 6 the titration behavior of β-C can be described in terms of its amino acid analysis and corresponds to a protein identical with β-B, except for the presence of two extra histidines, below that pH the protein passes through a region (between pH 6 and 4.5) in which it binds progressively fewer protons than expected, until below pH 4.5 the data can be described exactly by the amino acid composition of β-B; i.e., below pH 4.5, the two extra histidines make no contribution to the titration curve. It is significant that between pH 6 and 4.5 β-C is known to undergo a conformational transition, as evidenced by optical rotatory dispersion data (28). This conformational transition evidently results in the removal from contact with solvent and, thus, protonation of two basic groups, which from comparison with the A and B variants, are quite probably the difference imidazoles.

The final point to be established was the presence in β-C of the two anomalous carboxyls found in β-A and β-B. This was done by comparing the experimental data above pH 6.5 with curves calculated for 48 and 50 ionizable carboxyls, respectively. These curves, shown by thd dot-dash and dashed lines of Fig. 4, respectively, deviate from the data points, which are found to follow a transition curve from 48 to 50 carboxyls. This shows that β-C, as well as β-A and β-B (4), undergoes the alkaline conformational transition resulting in the unmasking to ionization of two side-chain carboxyls.

The above comparison of the experimental data with various calculated titration curves leads to the conclusion that the proton binding properties of β-lactoglobulin C can be described best in terms of the number of groups and pK's shown in Table I. Two sets of groups are abnormal: the two buried carboxyls and two basic groups, probably histidines which become protonated normally with a pK' of 7.25, but appear to lose their protons between pH 6 and 4.5. This pair of anomalous basic groups is not found in the other genetic variants. Curves were calculated for several other variations in the numbers of titratable groups. None, however, could describe the present data, which shows that the above analysis is unique.

In order to unmask the anomalously titrating basic groups, β-lactoglobulins A, B, and C were denatured either by exposure to pH 11.1 for 10 minutes or by solution in 8 M urea, and their titration behavior was determined. The results obtained with the alkaline denatured proteins are shown in Fig. 5. No points were taken between pH 3.7 and 5.9

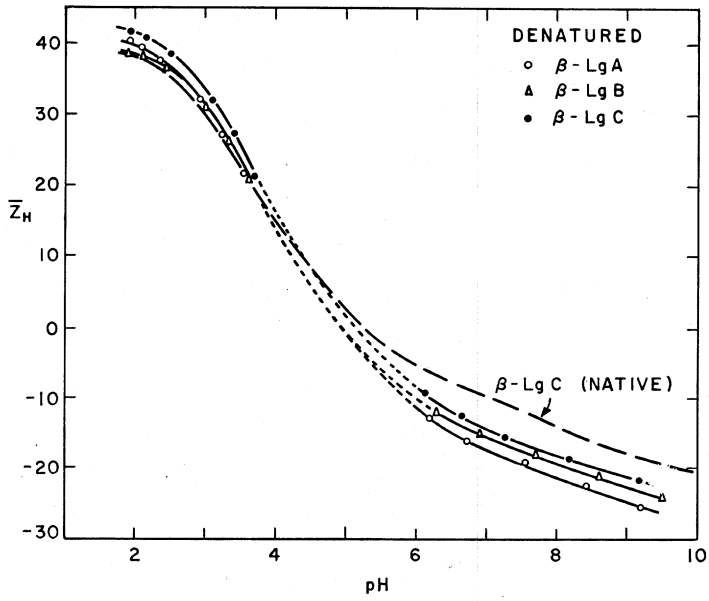

FIG. 5. Titration curves of alkali denatured β-lactoglobulins. The dashed line is the titration curve of native β-C.

due to the insolubility of the denatured proteins. In the pH region above the isoelectric point, the results for all three genetic variants are quite similar to those reported previously for a mixture of β-A and β-B (4). The steepening of the neutral region of the titration curve has disappeared and the points shift to values that are quite consistent with the presence of 50 normally titrating side-chain carboxyls in β-B and C and 52 such groups in β-A; the electrostatic interaction parameter, w, differs from that of the native protein, due to expansion of the denatured molecules. In addition, above pH 6, β-C binds two more protons due to its two extra histidines. Thus, on the alkaline side, the same differences are maintained between the three proteins in the denatured state as are found in the native material. In the acid region, the titration curve of denatured β-C is different from those of denatured β-A and β-B; it is found now that two additional groups are being titrated in denatured β-C, and the acid-binding capacity of the denatured C variant tends toward 42, i.e., as would be expected from its amino acid composition. Variants A and B tend toward a maximum proton binding of 40, just as in the native state. The deviation

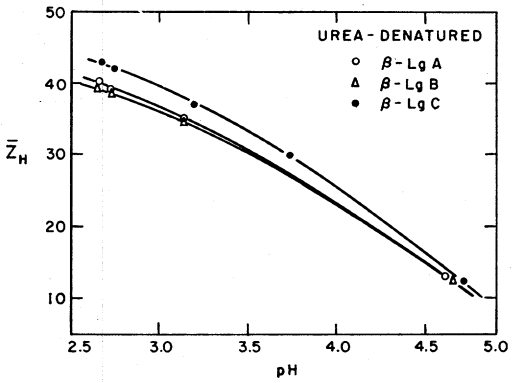

FIG. 6. Titration curve of urea denatured β-lactoglobulins.

of denatured β-A points from those of β-B reflects most probably a higher disorganization of the A variant on denaturation. The titration curve of native β-C has been drawn on the same figures as a dashed line. Comparison with the data on the denatured proteins reveals that, in the acid region the effective radius and charge distribution of β-B have not changed drastically as a result of alkaline denaturation, while those of β-A and β-C have been altered more. In the alkaline region, the structures of the three proteins have been changed equally and con-

siderably as evidenced by the equally large changes in w that can be deduced from the parallel shift of the three titration curves.

Figure 6 shows the results of titration of the three proteins in the presence of 8 M urea. Here the values of $\bar{Z}_H$ have only relative significance since the isoionic points in 8 M urea are not known (for purposes of plotting the data it was assumed that the values were the same as those in the native state, probably a poor assumption). Again, in the denatured proteins, the titration points of β-C fall on a curve different from those of β-A and β-B, revealing the titration of two more cationic groups per molecule in β-C than in the other two variants. Thus, urea denaturation, as well as alkaline denaturation, allows normal titration of all the ionizable groups known to be present from amino acid analysis.

DISCUSSION

The titration data on β-lactoglobulins A, B, and C are in excellent agreement with the studies of Tanford *et al.* on a β-AB mixture, as well as on β-A and β-B separately (2–4). All three proteins have one abnormal carboxyl per chain which ionizes only after the molecule undergoes a reversible conformational change with a pK of about 7.5.

The titration curve of β-C establishes beyond doubt that it has the same number of ionizable carboxyls as β-B, proving that the substitution of the histidine residues in the amino acid sequence of β-C is for glutamine residues in β-B, and not glutamic acid. This substitution is the more reasonable one to expect from the genetic code, since it requires the substitution of only one base in the sequence of the DNA triplet (29). Thus, we have β-B → β-C:gln → His:CAA or G → CAU or C (Glu → His would have required :GAA or G → CAU or C). In this connection, it is interesting to note that the other known substitutions in the β-lactoglobulins follow a similar pattern of single base substitution: β-B → β-A:Gly → Asp and Ala → Val:GGU or C → GAU or C and GCU or C → GUU or C. Thus, there is no need to postulate the existence of a β-AB hybrid molecule as has been proposed (30). Such a hybrid molecule has never been found; heterozygous animals have always been found to produce a mixture of the corresponding two β-lactoglobulins in approximately equal amounts and in accordance with simple Mendelian genetics (12).

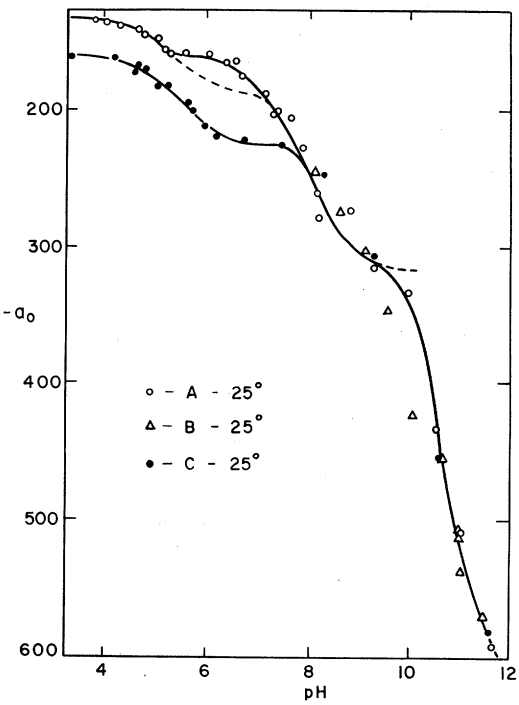

FIG. 7. Change with pH of the optical rotatory dispersion a_0 parameter for the β-lactoglobulins. The dashed line between pH 5 and 7 represents the best curve drawn through the β-B data (the actual points are omitted).

The data and results described here suggest that, during the conformational transition between pH 4.5 and 6, there are no major changes in the overall structure of the molecule. This is indicated by an insignificant change in w', and hence b, which is in contrast with the situation occurring in serum albumin (31). Figure 7 illustrates that a conformational transition occurs in this pH region for all three variants. In β-C this is accompanied by a change in the titration curve; in β-A and β-B no such change is observed. While the significance of this transition is discussed in detail elsewhere (28), the similarity of the optical rotatory properties of the three proteins in this pH region and the difference in their titration curves suggest that the anomalously titrating group in β-C is the difference histidine. In addition,

the further transition in a_0 above pH 6 for all three variants (4, 28, 32) reflect the conformational change which releases the anomalous carboxyls to ionization.

The removal of two titratable groups from protonation below pH 4.5 can be the result of two possible mechanisms. In the first, the conformational transition would be accompanied by the transfer of a protonated histidine residue from the surface of the molecule where it is exposed to solvent into the hydrophobic interior of the protein molecule. Such an event would have to be accompanied by the forced dissociation of histidine to the neutral state, since the burial of a single charged residue in the low dielectric interior of the protein molecule would require the expenditure of as much as 70–100 Kcal per mole of stabilization energy (33). This is prohibitive in view of the fact that the total net free energy of stabilization of a protein such as β-lactoglobulin is of the order of 10–15 Kcal per mole (34). In the second mechanism, the conformational transition would be accompanied again by the burial of the histidine, but now it would be carried into the hydrophobic interior in the protonated state as half of an ion pair; the anion could be a carboxylate ion which also became buried during the transition and which would remain unprotonated at low pH's. Such a process would not require the expenditure of much energy, since the ion pair would be buried essentially as a neutral entity and would possibly constitute a structure-stabilizing internal salt bridge.

The conformational transition of all three β-lactoglobulins between pH 4.5 and 6 has been described by a transition from the native (N) form of the protein to the acid (Q) form (28). In the case of β-C, this transition is triggered by the protonation of one carboxylic group per 18,000 dalton chain. The thermodynamic parameters of the $N \rightarrow Q$ transition and those of side-chain carboxyl ionization in β-lactoglobulin have been used to show that the enthalpy of the removal of the cationic group from contact with water is −4.1 Kcal per mole. The entropy of the same process, ΔS_{cat}, is given by $\Delta S_{cat} = \Delta S^* - \Delta S^\circ - \Delta S_{COOH}$, where ΔS^* is the overall entropy of the $N \rightarrow Q$ transition, ΔS° is the entropy of the conformational change alone with all key groups protonated at both ends of the reaction, and ΔS_{COOH} is the entropy of protonation of the carboxyl. Using the known values of these quantities, we have $\Delta S_{cat} = 24 - 20 - 18 = -14$ Kcal per deg-mole. The corresponding free energy, ΔF_{cat}, is found to be 0.2 Kcal per mole. The implications of these thermodynamic parameters for the two proposed mechanisms of burial of the cationic residue will be discussed in turn. Looking at the group burial mechanism first, these parameters can be regarded as those of the dissociation of the imidazole in the buried state. Comparison with the thermodynamic parameters of a normal histidine residue in a protein, the shift of a histidine pK close to zero ($\Delta F = 0.2$) results in the loss of 9 Kcal per mole of stabilization free energy; this is accompained by a loss of 11 Kcal per mole of enthalpy and a gain of 6 entropy units. Such values are not inconsistent with the presence of a predominantly hydrophobic effect (34–36). If the process involves the formation of a salt bridge between a histidinium and a carboxylate ion, the thermodynamic parameters, ΔF_{cat}, ΔH_{cat}, ΔS_{cat}, can be regarded as those of the ion pair formation. Both their signs and magnitudes are in quite reasonable agreement with values reported for the formation of ion pairs (37). The free energy loss of 6.5 Kcal per mole, due to the displacement of the carboxyl pK to close to zero, could be compensated by the structural stabilization resulting from the formation of an internal ionic bond. The present analysis cannot lead to the selection of either mechanism in preference to the other, and experiments aimed at the elucidation of this question are now in progress in our laboratory.

The decrease in pH from about 5.4 when salt is added to the ion-free isoionic proteins has been interpreted by Nozaki *et al.* (2) in terms of preferential binding of K^+ to the β-lactoglobulins. Recently, Baker and Saroff (38) have questioned this interpretation on the basis of direct binding experiments carried out with 0.5 M Na^+ above pH 5.8. These two apparently contradictory sets of results (2, 38) may be reconciled from the consideration of several factors. First, the binding studies of Baker and Saroff were carried out at pH's alkaline to the pH 4.5–6 conforma-

tional transition. It is possible that the K^+ binding properties of the β-lactoglobulins are altered by the conformational change. Nozaki *et al.* have postulated that the cations are bound at a carboxyl-rich locus. That such a cluster of carboxyls exists in the pH 4–5 range can be inferred from the low temperature titration of β-lactoglobulin A (39).

The observed drop in pH of the isoionic protein upon addition of salt could also result from a specific distribution of the discrete changes on the surface of the molecule in a configuration significantly different from the smeared charge model assumed in Eqs. 1–3. Tanford and Kirkwood (33, 40) have shown that variation in the distribution of charges can lead both to an increase and a decrease in the pK values determined with Eq. 3, even after correction of the experimental data for binding of ions other than protons; thus (41)

$$pK_0 = pK_0^\circ + 0.434 \frac{(\epsilon)\Delta\Psi_0}{kT}, \quad (4)$$

where pK_0 is the experimental pK after correction for ion binding (2), pK_0° is the intrinsic dissociation constant, ϵ is the electronic charge, and $\Delta\psi_0$ is the sum of the electrostatic interactions between the ionizable group in question and all other charged groups in the protein molecule at a net average charge, $\bar{Z}$, of zero. In terms of this interpretation, the data of Fig. 1a might indicate a decrease in pK_0 of carboxyls as salt is added (41). The direction of this change in pK is consistent with the low temperature titration of β-A (39), as well as the high dipole moment of this protein (42, 43) and its solubility as a function of ionic strength (44).

A third possible explanation of the observed drop in pH of the iosionic protein upon addition of salt could be that the conformational transition is dependent on $\Gamma/2$. In this case, addition of salt would displace the equilibrium toward the N form, since the $Q \rightarrow N$ transition is accompanied by the release of one proton per polypeptide chain (28). While no effect of ionic strength on the transition was noticed in the range of 0.03–0.3, the nature of the ion does affect the pK of the transition; this would imply that ions are bound to the protein below pH 6, resulting in the displacement of the transition. Experiments presently under way in our laboratory should help clarify this question.

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